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OBSERVATIONS ON THE GROWTH OF THE GONOCOCCUS AND THE STAPHYLOCOCCUS ALBUS FROM THE URETHRA IN PLATE CULTURE (A CRITICISM OF WARDEN'S WORK)*

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The identity of some well known bacteria is being doubted by progressive bacteriologists. Variations in the so-called "characteristic properties," which serve as criteria in the differentiation of bacteria, suggest the possibility that our predecessors have confused bacteriologic terminology by assigning names to many variations of a few bacteria, when growing in widely dissimilar environment. Much valuable knowledge will undoubtedly be obtained by a review of the subject of bacteriology in the new light. Yet this must be done with care. The Gram stain, a variable quantity in itself, and the elusive phantom, morphology, cannot give conclusive evidence that two bacteria, heretofore considered different, are the same, as has been contended in Warden's publications of last year, which we are about to discuss.

Warden,[1] in his studies of the gonococcus and the staphylococcus albus from the urethra, concludes that "Many, if not all, of the gram-negative, intracellular, biscuit- or coffee-bean shaped cocci, observed in the purulent discharge in acute gonorrhea, which are regarded as gonococci, and which serve as criteria of diagnosis, are not gonococci, but belong to the staphylococcus group. The provisional name of *Staphylococcus urethrae* is suggested for this coccus."

As a basis for this conclusion, Warden cites his observations on the Gram staining properties of the staphylococcus albus, obtained from urethral pus: (1) when grown on the usual media in pure culture and in mixed culture with the gonococcus; (2) when within leukocytes, in phagocytosis experiments made to obtain intracellular organisms, and (3) when in colonies.

Pure cultures of the staphylococcus albus and gonococcus were grown on plate culture media spread in thin films on microscopic slides which permitted staining and study of colonies *in situ*.

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* Received for publication, May 28, 1914.

[1] *Jour. Infect. Dis.*, 1913, 13, p. 124.

Further peculiarities in Gram staining, together with changes in morphology of the same staphylococcus were observed when the organisms were in suspension in body fluids such as blood-serum, hydrocele fluid and ascites fluid; and in ferments, such as dilute pancreatin, leukocytic extract and gonococcus autolysate. These fluids are of significance in the study because the product of dead leukocytes or leukocytic extract, and the by-product of gonococcus growth, gonococcus autolysate, are present within the urethra in urethritis.

Briefly, Warden's observations are as follows:

1. In suspensions of staphylococci, the Gram staining properties of the cocci vary according to the nature of the fluid from which the suspension is made. Coverglass preparations made from this coccus in normal salt solution show the familiar gram-positive staphylococci, even in suspensions which have stood at room temperature for weeks. But old suspensions three to six hours old in serum, hydrocele fluid, ascites fluid, weak pancreatin solution and gonococcus autolysate show gram-negative forms of the still viable cocci.

2. The intracellular diplococci in gonorrheal pus are probably gram-negative staphylococci in diplococcal form. Phagocytosis experiments demonstrate both the ease with which staphylococci, especially when in the gram-negative state, are taken up by leukocytes, and the difficulty experienced in ingesting gonococci. Even though the same conditions exist in the urethra as in the laboratory, the gonococcus is not an organism to be expected within the cells in gonorrheal pus.

3. The staphylococcus grows for the first few hours as a gram-negative organism. When staphylococci are planted on plate slides and stained at various stages of incubation, small, young colonies, up to 8 to 12 hours of age, all show gram-negative cocci. Older colonies show gram-negative bases, where the cocci are still in contact with the culture media, but in those portions of the colony which are elevated above the surface of the media, the cocci are gram-positive.

Before proceeding to a discussion of these findings, I wish to mention some general considerations pertinent to the cultivation of the gonococcus and the staphylococcus albus, some of which have been discussed in Warden's articles.

Staphylococcus albus and gonococcus colonies, when grown on the usual media, are unmistakably different. Microscopically, the large, slightly elevated, whitish colonies, which are seen at the end of eighteen to twenty-four hours incubation, are in no way to be confused with the smaller, transparent, dew-drop colonies of the gonococcus. Colonies of gonococci more than twenty-four hours old become more opaque, but never white to such an extent that one would confuse the growth with that of the staphylococcus on the same media. Microscopically, the two types of colonies are distinguishable. These small colonies are best compared when the mixed suspensions of gonococcus and staphylococcus are planted on slide-plate media, such as Warden uses, and stained *in situ*. Eight- to twelve-hour growths are best for the purpose, since the colonies at this time are composed of but from one to three layers of bacteria. The staphylococcus colony appears as an irregularly bunched, elevated colony of cocci and diplococci, from two to three layers thick at the center to a single layer at the periphery, with coccal forms predominating. The gonococcus colony is smaller, flat and composed chiefly of diplococci arranged mosaically over the surface of the media in a single layer. Both types of colonies are gram-negative at this stage of incubation, but in spite of the similarity, are easily distinguishable by the arrangement of the cocci composing them.

Pure cultures of the gonococcus are seldom obtained from pus in gonorrheal urethritis. Pus from early cases of urethritis, from two to six days after the appearance of the discharge, usually shows a preponderance of gonococcus colonies and a much smaller number of staphylococcus colonies. In the late cases, two or more weeks from the appearance of the discharge, the growth is chiefly staphylococcus with a few gonococcus colonies.

Diplococcal forms are common in any rapidly growing coccus. The phenomenon is due to rapid growth with incomplete division at the time of observation. Perhaps in the best coverglass preparations, morphology may be sufficient for differentiation of these cocci from gonococci, but this is not the case where the bacteria are stained on plate culture media, as in Warden's experiments, or clinically, where other bacteria and much cell detritus is present.

The Gram method of staining bacteria is not absolutely reliable, and numerous variations in the reaction of the same bacteria in changed environment have been recorded. Eisenberg[2] has shown that gram-positive bacteria undergoing spontaneous degeneration, a process of bacteriolysis, become gram-negative. Also gram-positiveness of bacteria is influenced by serum without the picture of bacteriolysis being presented. The cocci are still viable. The causal agent is not mentioned.

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[2] *Centralbl. f. Bakteriol.*, Abt. 1, O., 1910, 56, p. 193.

Schmidt,[3] working with colon bacilli found the organisms gram-positive when occurring in fat stools. Cedarkreutz[4] found some cocci and colon bacilli gram-positive in butter. Nikitin[5] found that by treating some varieties of bacteria with fat-splitting ferments they became gram-positive. Grimme[6] records the observation that weak alkalies, 5 percent hydrochloric acid, pepsin and trypsin will produce gram-negative bacteria.

Eisenberg[7] expresses the opinion, from observation of the work of others as well as from his own study, that introduction of albuminous material into bacterial preparations and culture media produces gram-negative bacteria in some cases.

Of these observations, that which most concerns the question in hand is the effect of albuminous material on the Gram stain. Inasmuch as the gonococcus is difficult to grow in culture, except on special media containing blood-serum or its equivalent, albumin is present in Warden's experiments wherever gram-negative staphylococci are seen. The same condition results in his suspensions of the staphylococcus in body fluids, but is absent in the normal salt suspensions in which the cocci remain gram-positive. Similarly, gonococcus autolysate contains albumin as a requisite to the growth of the gonococcus which produces it. Experimental evidence has already been indicated to show the possibility of ferments being further factors in producing gram-negative bacteria in this case.

At the suggestion of Dr. H. Cabot and under the supervision of Dr. J. H. Wright, I have endeavored to repeat Warden's experiments exactly as described by him with the exception of two minor variations from his technic, viz.: (1) hydrocele agar was substituted for ascitic agar, since our cultures grew better on this media, as we prepare it in this laboratory, than on our ascites fluid agar; (2) the substitution of Bismarck brown for 1 percent carbol fuchsin as a counterstain.

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[3] Cited by Eisenberg.[7]

[4] *Arch. f. Dermat. u. Syph.*, 1908, 93, p. 355.

[5] *Arch. Russ. de Pathol.*, 1908, pp. 132, 162.

[6] *Centralbl. f. Bakteriol.*, Abt. 1, O., 1902, 32, p. 1.

[7] *Centralbl. f. Bakteriol.*, Abt. 1, O., 1910, 56, p. 193.

Our findings are essentially the same as those reported by Warden. gram-negative staphylococci are readily produced by treatment of gram-positive organisms with serum or body fluids, and by the ferment action of leukocytic extract, gonococcus autolysate, and pepsin. By phagocytosis of these gram-negative staphylococci, leukocytes containing organisms similar to those found in gonorrheal pus were produced. But gram-negative staphylococci in cells were not sufficiently characteristic to deceive the house officers of this hospital, and the diagnosis of gonorrhea was not once made. Gonococci, also, can be taken up by leukocytes, *in vitro,* but less readily than gram-negative staphylococci.

On plate cultures the staphylococcus grows in gram-negative form in cultures up to eight hours; then the cocci become gram-positive in all portions of the colony where the individual organisms are not in contact with the surface of the media, i. e., in that part of the colony which is elevated above the surface of the media.

Further experimentation with plate cultures revealed significant facts. A suspension of the same staphylococcus was made in normal salt solution. Coverglass preparations from this suspension showed all gram-positive cocci. With material from this suspension, plates were prepared and stained immediately without incubation. The cocci on the plate were gram-negative. If, however, the bacteria from another slide, like the first, be washed off into salt solution again, they stain gram-positive as before. Gram-negativeness appears to depend entirely on the presence of the staphylococci on the plate media. Staphylococcus albus and aureus from abscess, and streptococci obtained from the throat, were also found to be gram-negative on plate media, but gram-positive in water or salt solution suspensions.

If a small colony from a six- to eight-hour slide plate culture of staphylococcus albus is removed from its bed and washed in normal salt solution and the plate stained as usual by Gram's method, and coverglass preparations, made from the suspension containing the colony, removed, the colonies remaining on the plate are all gram-negative and the cocci in suspension gram-positive. These facts lead one to doubt that the staphylococcus normally grows gram-negative for a time, but to believe rather that the appearance of gram-negativeness is due to an artefact. If so, what is the causal factor? Extracts of hydrocele agar, which contain phosphates and other salts, do not produce gram-negativeness. Thin films of celloidin, when replacing

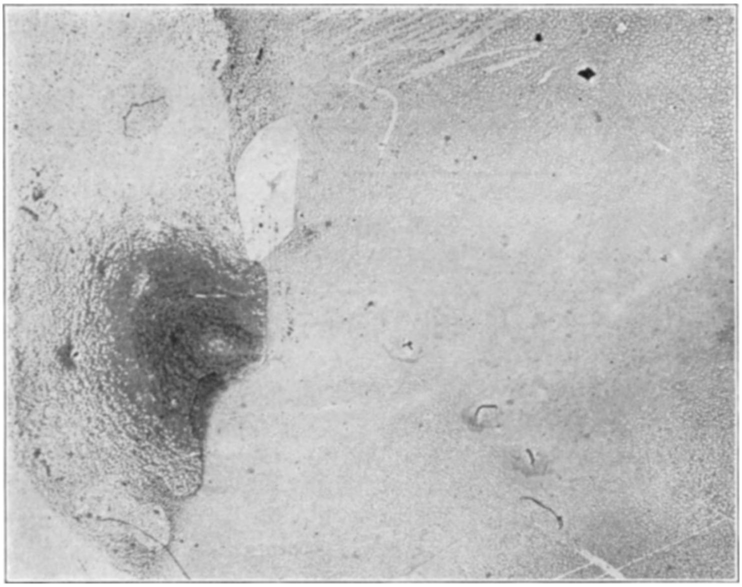

Fig. 1.—Low magnification (x12) of a field of *Staphylococcus albus* with the right half of the field coated with egg albumin. Cocci to the left of the albumin line are gram-positive, those to the right gram-negative. Field shown in Figure 2 indicated at X.

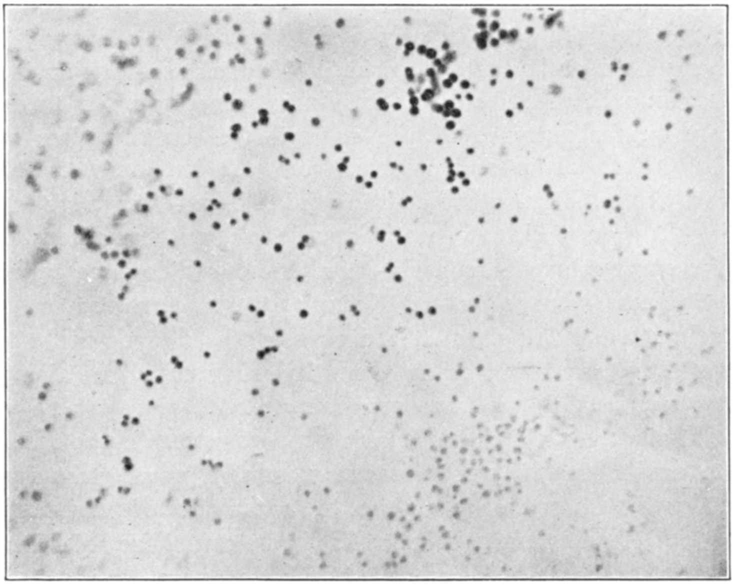

Fig. 2.—Edge of albumin field. Cocci to the left are not covered by albumin and stain gram-positive. The right half of the field shows gram-negative cocci beneath the albumin. x1,500.

agar on plate slides, cause no loss of gram-staining ability. Variations in the time alloted to each step in the performance of the stain makes no essential difference in the results, except that in some cases where methyl violet has been allowed to remain in contact with the bacteria for a considerable time, decolorization is difficult and the bacteria show dark centers with gram-negative peripheries.

While working with suspensions of staphylococci in hydrocele fluid which contained small flakes of albuminous material, I observed that in coverglass preparations all the cocci lying free in the field were gram-positive, while those beneath the flakes were gram-negative. Also, in suspensions made in blood-serum which contained a few red cells in clumps, the cocci which were entangled in cell clumps and fibrin were gram-negative, but elsewhere gram-positive. These observations were made in newly prepared suspension where no incubation had taken place, nor had the preparations been allowed to stand for some hours — two conditions which Warden considers essential for the production of gram-negative staphylococci in serum. Albumin clearly interferes with the staining properties of these organisms. Experi-

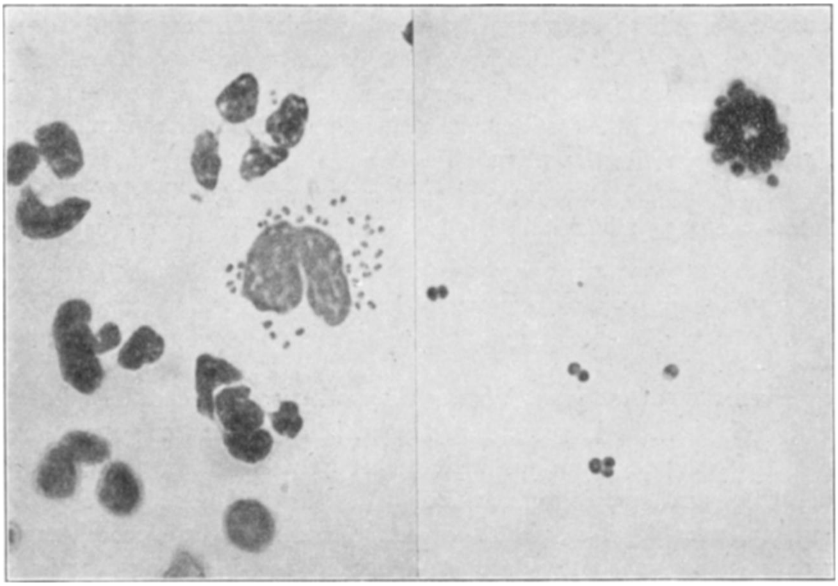

Fig. 3.—Left half of field shows intracellular gonococci from urethral pus. Right half of the field shows phagocyted gram-negative staphylococci. x1,500.

mental evidence tends to show that by long contact with serum, the bacteria become sufficiently coated with albumin to respond abnormally to the Gram stain. That there is no permanent change in the bacteria themselves is demonstrated by the fact that after washing in salt solution they again become gram-positive. Gram-negative cocci are viable since cultures from suspensions in which gram-negative forms predominate show abundant growth.

Mechanical interference by albumin clearly explains the growth of gram-negative colonies from gram-positive cocci. A thin layer of albumin, derived from the media, covers the surface of the media to a sufficient depth to submerge the small colonies, but apparently does not in any way interfere with growth. When the colony attains sufficient size to rise above the high-water mark of albumin, the top stains gram-positive while the portion remaining below the albumin continues to stain gram-negative.

Gram-negative staphylococci can be prepared at will. If a dilute solution of egg albumin is applied to a portion of the surface of a coverglass preparation of staphylococci with a camel's-hair brush, and the Gram stain applied, it is found that in the portion of the preparation covered by albumin the cocci are gram-negative, elsewhere they are gram-positive.

Even though we grant Warden's hypothesis that conditions found within the urethra in gonorrheal urethritis, and laboratory conditions are essentially the same, we believe that he produces insufficient evidence for the sweeping conclusion that, "Many, if not all, the intracellular, gram-negative, biscuit- or coffee-bean shaped diplococci, commonly called gonococci," are staphylococci masquerading in gram-negative form.

He believes that gram-negative diplococcal forms of staphylococci are indistinguishable from gonococci when seen in the ordinary coverglass preparations from pus.

By phagocytosis experiments he shows that the gram-negative staphylococcus is more likely to be the organism within the pus cell than the gonococcus. As a further argument he states that the staphylococcus normally has a gram-negative stage of growth eight to twelve hours long, which stage is the one seen in acute gonorrhea. The chronic gonorrheas represent late stage gram-positive growth of the same organism.

He has failed to note the fact that the Gram method of staining bacteria does not give uniform results when applied to bacteria in unusual surroundings, especially in the presence of fats and albumin. The Gram stain, although supported by the doubtful aid of morphology, does not warrant the reliance placed on it.

He has also entirely neglected cultural evidence of the presence of the gonococcus in gonorrhea. The large proportion of gonococcus colonies and the few staphylococcus colonies seen in cultures taken directly from urethral pus in early cases of urethritis, seems to indicate beyond doubt that the intracellular diplococci "commonly called gonococci" and which produce gonococcus colonies, are not staphylococci; and that the reason so few staphylococcus colonies appear is that few are present in the pus. If the intracellular organisms are viable gram-negative staphylococci, which laboratory experiments show to be possible, there should be a growth of almost pure staphylococcus instead of gonococcus colonies. We have absolutely no evidence to show that staphylococci in their gram-negative state ever produce the gonococcus type of colony, or organisms which behave in culture like the gonococcus. The staphylococci present in acute urethritis pus may be in gram-negative form; indeed, there is reason to believe that they are gram-negative in pus with the high albumin content of acute urethritis pus.

I have shown that the staphylococcus does not have a short gram-negative stage of growth, but that this is an illusion produced by mechanical interference of albumin with the staining reaction of the bacteria, either by preventing penetration of the basic stain or interference with fixation of the stain. Plate culture media, which contains considerable albumin in added serum, hydrocele or ascitic fluid necessary to the growth of gonococci, and used by Warden for the staphylococcus also, is covered over with a thin layer of albumin deep enough to cover the small colonies completely and the bases of the larger ones producing a gram-negative reaction in these portions.

The morphology of cocci and diplococci, except perhaps in cover-glass preparations from pure culture, gives little aid in differentiation and is an even less valuable aid in pus with cell detritus. I have been unable to produce "pus cells" containing gram-negative diplococca staphylococci which are indistinguishable from gonorrheal pus although the resemblance was close in some cases.

SUMMARY

Gram-negative diplococcal staphylococci may be produced in the laboratory, and it is conceivable that they also occur in the urethra, but gram-negativeness is due to mechanical interference of albumin with the Gram stain.

The staphylococcus albus has no stage of growth in which it is a gram-negative organism. The appearance is due to an artefact.

The evidence presented by Warden that gonorrheal urethritis is due to the staphylococcus albus is based on erroneous interpretations of his observations and is fallacious.